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THE REGULATION OF GROWTH PLATE CHONDROCYTES BY TRANSFORMING
GROWTH FACTOR- β AND ITS MECHANISM OF ACTION

A
THESIS

Presented to the Faculty of
The University of Texas Graduate School of Biomedical Sciences
at San Antonio
in Partial Fulfillment
of the Requirements
for the Degree of
MASTER OF SCIENCE

By
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San Antonio, Texas

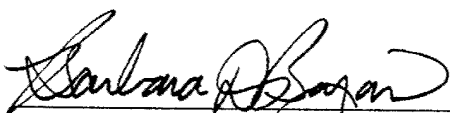
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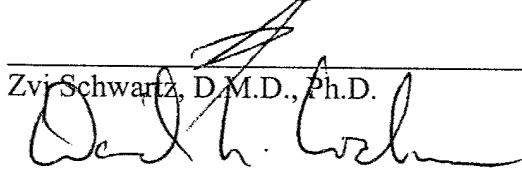
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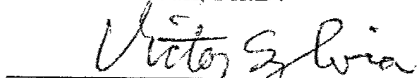


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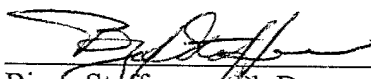
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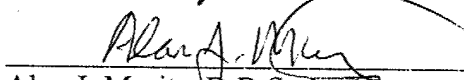
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DEDICATION

I would like to dedicate this thesis to my lovely wife [REDACTED], my children [REDACTED] [REDACTED], and my parents [REDACTED] [REDACTED]. Thank you [REDACTED] for your unconditional support, your understanding and love. Thank you [REDACTED] for filling my long days and nights with laughs and kisses. Thank you “Mami” and “Papi” for inspiring me early on with high values, intellectual curiosity, and love for hard work.

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THE REGULATION OF GROWTH PLATE CHONDROCYTES BY
TRANSFORMING GROWTH FACTOR- β AND ITS MECHANISM OF ACTION

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Transforming growth factor- β 1 (TGF- β 1) regulates chondrocytes through Smad protein-mediated mechanisms, and has been shown to increase PKC. To test whether other signaling pathways play a role, this study examined if the physiologic response of rat costochondral growth zone (GC) chondrocytes to TGF- β 1 is through TGF- β 1 type II or III receptors and also the contribution of protein kinase C (PKC), protein kinase A (PKA), and G-proteins to this process. Growth zone chondrocytes

were isolated from rat costochondral cartilage and cultured. Cell cultures were incubated with 0.11, 0.22, or 0.88 ng/ml TGF- β 1 \pm 0.5, 1 or 2 μ g/ml anti-type II or III receptor antibodies, or 25, 50 or 100 μ g/ml soluble type II or III receptors for 24 hours. Cells were cultured in the presence or absence of PKC inhibitors chelerythrine, staurosporine, and H-7; the PKA inhibitor H-8; cyclooxygenase (COX) inhibitors indomethacin, resveratrol, and NS-398; the PKC activator: diacylglycerol; the G protein activator GTP γ S; and G protein inhibitors pertussis toxin, cholera toxin, and GDP β S, and physiological responses of the cells examined as well PKC activity.

Treatment of confluent growth plate chondrocytes with TGF- β 1 stimulated [3 H]-thymidine and [35 S]-sulfate incorporation as well as alkaline phosphatase and PKC specific activities. The receptor responsible for TGF- β 1-dependent PKC and the physiological response of GC cells to TGF- β 1 was tested using anti-type II TGF- β receptor antibody and soluble type II TGF- β receptor. The results showed that TGF- β 1 mediates these effects through the type II receptor. Inhibition of PKC with chelerythrine, staurosporine, or H-7 caused a dose-dependent decrease in these parameters, indicating that PKC signaling was involved in the physiological response of the cells to TGF- β 1. The increase in [3 H]-thymidine incorporation and alkaline phosphatase specific activity were also regulated by protein kinase A (PKA) signaling, since the effects of TGF- β 1 were partially blocked by the PKA inhibitor H-8.

The mechanism of TGF- β 1 activation of PKC is through phospholipase A₂ (PLA₂). Arachidonic acid increased PKC in control cultures and was additive with TGF- β 1. Prostaglandins are required, as indomethacin and the COX-1 inhibitor resveratrol blocked the effect of TGF- β 1, but the COX-2 inhibitor NS-398 had no effect on the TGF- β 1-dependent activity. TGF- β 1 stimulated prostaglandin E₂

(PGE₂) production and exogenous PGE₂ stimulated PKC, but not as much as TGF- β 1, suggesting that PGE₂ is not sufficient for all of the prostaglandin effect. In contrast, phospholipase C (PLC) is not involved. TGF- β 1 was not regulated by diacylglycerol; neither dioctanoylglycerol nor inhibition of diacylglycerol kinase with R59022 had an effect. G-proteins mediate TGF- β 1 signaling at different levels in the cascade. TGF- β 1-dependent increases in PGE₂ levels and PKC were both augmented by the G protein activator GTP γ S, whereas inhibition of G-protein activity via GDP β S, pertussis toxin, or cholera toxin blocked stimulation of PKC by TGF- β 1, indicating that both G_i and G_s are involved. Inhibition of PKA with H-8 partially blocked TGF- β 1-dependent PKC, suggesting that PKA inhibition on the physiological response was through PKA regulation of PKC signaling. The effects of TGF- β 1 on PKC are not mediated by a direct effect on preexisting membrane-associated PKC. There was no change in PKC specific activity of isolated matrix vesicles or plasma membranes that were incubated directly with TGF- β 1.

The results of this study confirm that the effects of TGF- β 1 on growth zone chondrocytes are mediated by at least three separate but interdependent pathways: PKC, PLA₂/arachidonic acid/prostaglandin, and PKA. This study concludes that TGF- β 1 binds to its type II receptor in the cell membrane, which phosphorylates and binds to the type I receptor. TGF- β 1 type I receptor activates Smad proteins, but also activates G-proteins, altering the activity of PLA₂, which produces arachidonic acid. Arachidonic acid is metabolized by cyclooxygenase to prostaglandins, which bind to EP1 and EP2 receptors on the growth zone chondrocyte cell surface. EP1 signaling stimulates PKC activity, while EP2 signaling increases cAMP, which activates PKA. This suggests that TGF- β 1 signals through PKC and PKA to increase alkaline phosphatase activity and to stimulate proliferation, and through PKC to stimulate proteoglycan production.

TABLE OF CONTENT

| | |
|--|-----------|
| Title | Page i |
| Approval | ii |
| Dedication | iii |
| Acknowledgement | iv |
| Abstract | v |
| Table of Contents | viii |
| List of Figures | x |
| I. INTRODUCTION AND LITERATURE REVIEW | 1 |
| II. MATERIALS AND METHODS | 5 |
| A. Reagents | 5 |
| B. Chondrocytes Cultures | 5 |
| C. Role of TGF- β 1 | 6 |
| 1. Cell Number | 7 |
| 2. [3 H]-Thymidine Incorporation | 7 |
| 3. [35 S]-Sulfate Incorporation | 7 |
| 4. Alkaline Phosphatase | 8 |
| D. Role of PKC in physiologic response of GC to TGF- β 1 | 9 |
| E. PGE $_2$ Production | 9 |
| F. Role of the Protein Kinase A | 10 |
| G. TGF- β 1-dependent Regulation of PKC | 10 |
| 1. Effect of Arachidonic Acid, Prostaglandins & PGE $_2$ | 11 |
| 2. Effect of Diacylglycerol | 11 |

| | Page |
|---|------|
| 3. Direct Effect of TGF- β 1 on Membrane Associated PKC | 12 |
| H. Statistical management of the Data | 14 |
| III. Results | 15 |
| A. Role of TGF- β 1 Receptors | 15 |
| B. Role of PKC | 25 |
| C. Mechanism of Action of TGF- β 1 | 34 |
| IV. Discussion | 42 |
| V. Literature Cited | 47 |
| VI. Vita | 52 |

LIST OF FIGURES

| | Page |
|---|------|
| Figure 1 Effect of TGF- β 1 type II receptor antibody on TGF- β 1-induced protein kinase C activity. | 17 |
| Figure 2 Effect of TGF- β 1 type II receptor antibody on TGF- β 1-induced [35 S]-sulfate incorporation | 18 |
| Figure 3 Effect of TGF- β 1 type II receptor antibody on TGF- β 1-induced [3 H]-thymidine incorporation | 19 |
| Figure 4 Effect of TGF- β 1 type II receptor antibody on TGF- β 1-induced alkaline phosphatase activity | 20 |
| Figure 5 Effect of TGF- β 1 type II receptor antibody on TGF- β 1-induced protein kinase C activity | 21 |
| Figure 6 Effect of TGF- β 1 soluble type II receptor on TGF- β 1-induced [35 S]-sulfate incorporation | 22 |
| Figure 7 Effect of TGF- β 1 soluble type II receptor on TGF- β 1-induced [3 H]-thymidine incorporation | 23 |
| Figure 8 Effect of TGF- β 1 soluble type II receptor on TGF- β 1-induced alkaline phosphatase activity | 24 |
| Figure 9 Effect of PKC inhibitor, chelerythrine, on TGF- β 1-induced [3 H]-thymidine incorporation | 26 |
| Figure 10 Effect of PKA inhibitor, H-8, on PKC activity in growth zone chondrocytes | 27 |
| Figure 11 Effect of PKC inhibitor, chelerythrine, on TGF- β 1-dependent [35 S]-sulfate I incorporation and alkaline phosphatase specific activity | 28 |
| Figure 12 Effect of PKC inhibitor, staurosporine, on TGF- β 1-dependent [3 H]-thymidine incorporation | 29 |
| Figure 13 Effect of PKC/PKA inhibitors, H-7 and H-8, on TGF- β 1-dependent [3 H]-thymidine incorporation | 31 |

| | Page |
|---|------|
| Figure 14 Effect of TGF- β 1 on PGE ₂ production | 33 |
| Figure 15 Effect of G-protein activation on TGF- β 1-dependent PGE ₂ production | 35 |
| Figure 16 Effect of COX inhibitors on TGF- β 1-dependent PKC activity | 37 |
| Figure 17 Role of G-proteins in TGF- β 1-dependent PKC activity using pertussis toxin and cholera toxin | 38 |
| Figure 18 Role of G-proteins in TGF- β 1-dependent PKC activity using GDP β S | 39 |
| Figure 19 Role of G-proteins in TGF- β 1-dependent PKC activity using GDP γ S | 41 |
| Figure 20 Proposed pathways of TGF- β 1 regulation of growth zone chondrocytes | 46 |

I. INTRODUCTION AND LITERATURE REVIEW

Transforming growth factor- β 1 (TGF- β 1) is a ubiquitous peptide hormone synthesized as an inactive 110 kDa homodimer, which is cleaved to the active 25 kDa homodimer by decreased local pH or proteolytic cleavage (Assoian *et al.*, 1983; Bonewald *et al.*, 1991; Pedrozo *et al.*, 1998). TGF- β 1 regulates the growth and differentiation of many cells. It can be a growth inhibitor or a growth promoter, depending on the cell type and conditions under which the experiment is conducted (Moses *et al.*, 1985). It was originally purified from demineralized bone and was assayed for activity by its ability to stimulate mesenchymal cells to differentiate into chondrocytes (Bonewald *et al.*, 1990, Schofield *et al.*, 1990).

In epiphyseal cartilage, TGF- β 1 is localized in the resting and upper hypertrophic zones of the growth plate. Similarly, the rat costochondral resting zone and growth zone (prehypertrophic and upper hypertrophic) chondrocytes synthesize latent TGF- β 1 (Boyan *et al.*, 1994) and latent TGF- β 1 binding protein-1 (LTBP1) both *in vivo* and *in vitro* (Pedrozo *et al.*, 1998). Latent TGF- β 1 is stored in the growth plate extracellular matrix as a 290 kDa complex consisting of latent TGF- β 1, and latent TGF- β 1 binding protein-1 (Pedrozo *et al.*, 1998).

Rat costochondral cartilage cells are particularly sensitive to TGF- β 1. Costochondral chondrocytes respond to active TGF- β 1 *in vitro* by increasing alkaline phosphatase activity in a biphasic manner (Schwartz *et al.*, 1998). In comparison to bone cells, the costochondral chondrocytes exhibit maximal differentiation responses to doses of 0.22 ng/ml, at higher concentrations of 0.88

ng/ml proliferation is affected, whereas 10-fold higher concentrations are required for osteoblasts to exhibit the same effect (Bonewald *et al.*, 1992). TGF- β 1 stimulates protein kinase C (PKC) activity in both resting zone and growth zone cells, but resting zone cells exhibit the increase more rapidly, suggesting a greater sensitivity to the growth factor. Although TGF- β 1 has been shown to act on chondrocytes, its precise mechanisms of action require clarification.

In an effort to elucidate the mechanisms by which TGF- β 1 acts, Smad proteins were identified six years ago as intracellular mediators of signaling by TGF- β family members (Zhang *et al.*, 1996). Intracellular signal transduction is initiated by binding of TGF- β ligands to specific type II receptors at the cell surface. These receptor-ligand complexes then recruit type I receptors in a ternary complex. Both receptor types contain an intracytoplasmic serine/threonine kinase domain, but the heteromeric protein kinase receptor has a limited ability to bind ligand; this limitation is overcome by the action of the TGF- β type III receptor (betaglycan). The type III receptor presents TGF- β directly to the kinase subunit of the signaling receptor (Lopez-Casillas *et al.*, 1993), forming a high affinity ternary complex. The ectodomain of the type III receptor can be released as a soluble proteoglycan (Lopez-Casillas *et al.*, 1991). This soluble TGF- β type III receptor may have a function in the localization and delivery or clearance of activated TGF- β (Boyd *et al.*, 1990). Once recruited in the complex, the type I receptor becomes phosphorylated by the type II receptor, and in turn transmits signals to the Smads proteins. A multitude of studies in cell lines has indicated that vertebrate Smad 2 and Smad 3 (R-

Smads) act in activin/TGF- β pathways (Heldin *et al.*, 1997). When fused to a well-defined heterologous DNA binding domain the MH2 domain of R-Smads displays a transcriptional activation activity in mammalian cells (Whitman 1998; Kawabata *et al.*, 1999). This, together with the observed ligand induced nuclear accumulation of Smad complexes, led to the idea that Smads might bind to DNA and act as transcription factors.

Apparently, other signaling pathways are involved. The effect of TGF- β 1 on proteoglycan production in resting zone chondrocytes is PKC-dependent, but inhibition of PKC does not affect the TGF- β 1-stimulated increase in proliferation or alkaline phosphatase activity (Schwartz *et al.*, 1998). Studies looking at the effects of TGF- β 1 in rat articular chondrocytes support the hypothesis that PKA mediates part of the TGF- β 1 response (Osaki *et al.*, 1999). These investigators used concentrations of H-7 that also block PKA, so it is not clear whether PKC or PKA was responsible for the TGF- β 1 effect. Specific inhibition of PKA also inhibits the effect of TGF- β 1 on proliferation and alkaline phosphatase in resting zone chondrocytes (Hu *et al.*, 1998). Also, TGF- β 1 has been shown to activate the MAP kinase pathway (Sylvia *et al.*, 1994, Schwartz *et al.*, 1998), and activation of MAP kinase is through PKC (Chaudhary *et al.*, 1998). These observations suggest that in addition to Smad proteins, surface receptor binding may be involved in the regulation of growth plate chondrocytes through PKC and PKA.

The purpose of this research is to determine whether the effect of TGF- β 1 on costochondral growth zone chondrocytes is mediated through the TGF- β 1 type II or

type III receptor, and to determine if there are any other signaling pathways involved.

II. MATERIALS AND METHODS

A. Reagents

The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): prostaglandin E₂ (PGE₂), indomethacin (a general cyclooxygenase inhibitor), and reagents for the alkaline phosphatase assay. The following chemicals were purchased from Calbiochem (San Diego, CA): 1,2-dioctanoyl-*sn*-glycerol (DOG), R59022 (diacylglycerol kinase inhibitor), chelerythrine, staurosporine, and H-7 (PKC inhibitors), H-8 (PKA inhibitor), arachidonic acid, pertussis toxin (G_i inhibitor), cholera toxin (G_s inhibitor), GDPβS (general G-protein inhibitor), GTPγS (G-protein activator), resveratrol (Cox-1 inhibitor), and NS-398 (Cox-2 inhibitor). PKC assay reagents and Dulbecco's modified Eagle medium (DMEM) were obtained from GIBCO-BRL (Gaithersburg, MD). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay reagent obtained from Pierce Chemical Co. (Rockford, IL). Recombinant human TGF-β1, anti-TGF-β1 receptor II and receptor III antibodies, and soluble TGF-β1 receptor II and receptor III were obtained from R&D Systems, Inc. (Minneapolis, MN). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). The PGE₂ radioimmunoassay kit, [³H]-thymidine, [³⁵S]-sulfate, and [³²P]-ATP were obtained from NEN-DuPont (Boston, MA).

B. Chondrocyte Cultures

The cell culture model used in this study has been previously described (Boyan *et al.*, 1988; Boyan *et al.*, 1992). Ribcages were removed from 125g Sprague-

Dawley rats and placed in Dulbecco's modified Eagle's medium (DMEM). Growth zone (GC) cartilage was dissected with the intervening tissue removed to maintain a distinct cell zone. The cartilage was sliced and incubated overnight in DMEM containing antibiotics at 37°C with 5% CO₂ in air and 100% humidity. After two 20-minute washes in Hank's Balanced Salt Solution (HBSS), the cells were released by sequential digestions using 1% trypsin for 1 hour and 0.02% collagenase for 3 hours. After complete digestion, the cells were separated from debris by filtration, collected by centrifugation at 500 x g for 10 minutes, resuspended in DMEM, and plated at a density of 10,000 cells/cm² for resting zone cells and 25,000 cells/cm² for growth zone cells. Cells were incubated in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin-fungizone, and 50ug/ml ascorbic acid (vitamin C) in an atmosphere of 5% CO₂ and 100% humidity at 37°C. At confluence (7-10 days), cells were subcultured using the same plating density and allowed to return to confluence. Third passage, confluent cultures were subpassaged into 24-well microtiter plates and grown to confluence. All experiments were confluent fourth passage cells. Previous studies have demonstrated a retention of differential phenotypic markers, both morphologically and biochemically, at this passage (Boyan *et al.*, 1988).

C. Role of TGF- β 1 Receptors

To determine whether the response of growth zone cells to TGF- β 1 involves the Type II or Type III TGF- β 1 receptor, cultures were incubated with 0.11, 0.22, or 0.88 ng/ml TGF- β 1 \pm 0.5, 1 or 2 μ g/ml anti-type II receptor or anti-type III receptor

antibodies, or with 25, 50 or 100 µg/ml soluble type II or III receptors for 24 hours. Changes in [³H]-thymidine incorporation, proteoglycan sulfation, and alkaline phosphatase specific activity were assessed. I did not specifically examine the role of the type I receptor, since antibodies to this receptor were not available. I did not include cultures treated with nonspecific IgG₁ because previous studies failed to show an effect on any of the parameters we examined (Sylvia *et al.*, 2000).

1. Cell number

At harvest, cultures were washed twice with DMEM and trypsin (1% in HBSS) was added. After 5 minutes the reaction was stopped with DMEM containing 10 % FBS. Cells were collected by centrifugation at 500 x g for 5 min, resuspended in saline, washed twice, and counted with an automatic Coulter counter (Beckman Instruments, Hialeah, FL).

2. [³H]-Thymidine incorporation

DNA synthesis was estimated by measuring ³H Thymidine incorporation into trichloroacetic acid-insoluble cell precipitates. Quiescence was induced by incubating confluent cultures for 48 h in DMEM containing 1% FBS. The medium was replaced with DMEM containing 1% FBS, TGF-β1 ± vehicles, and the appropriate concentration of soluble receptor or anti-receptor antibody. Two hours before harvest, [³H]- thymidine was added. Radioactivity in trichloroacetic acid-precipitable material was measured by liquid scintillation spectrometry.

3. [³⁵S]-Sulfate incorporation

Proteoglycan synthesis was assessed by measuring [³⁵S]-sulfate incorporation by confluent cultures of fourth-passage chondrocytes according to the method of

O'Keefe *et al.*, 1988. At confluence, fresh medium containing vehicle \pm TGF- β 1 \pm soluble receptors or anti-receptor antibodies was added to the cells and the incubation continued for an additional 24 hrs. Four hours before harvest, 50 μ l DMEM containing 18 μ Ci/ml [35 S]-Sulfate and 0.814 mM carrier sulfate was added to each culture. At harvest, the conditioned media were removed, the cell layers collected and the amount of [35 S]-Sulfate incorporated determined by liquid scintillation spectrometry. The protein content was determined using the BCA protein assay and the data expressed as disintegrations per minute per mg protein in the cell layer.

4. Alkaline phosphatase

Alkaline phosphatase activity was assayed in cell layer lysates as the release of p-nitrophenol from p-nitrophenylphosphate at pH 10.2 (Bretaudiere and Spillman, 1984) and specific activity calculated. Cell layers were prepared following the method of Hale *et al.*, 1986. At harvest, culture media was decanted and the cell layers were washed twice with PBS before removal with a cell scraper. After centrifugation, the cell layer pellets were washed once more with PBS and suspended by vortexing in 500 μ l deionized water plus 25 μ l 0.05% Triton-X-100. Enzyme assays were performed on these lysates after further disruption by freeze/thawing three times. Isolated cells were harvested as described above for the determination of cell number, except that after the cell pellets had been washed twice with PBS, the cells were resuspended by vortexing in 0.5 ml of deionized water with 25 μ l of 1% Triton-X-100. Cultures were treated with 0.11, 0.22, or 0.88 ng/ml TGF- β 1 \pm soluble receptors or anti-receptor antibodies.

D. Role of PKC in Physiologic Response of Growth Zone Chondrocytes to TGF- β 1

To determine whether the response of growth zone cells to TGF- β 1 involves PKC, cultures were incubated with 0.11, 0.22, or 0.88 ng/ml TGF- β 1 \pm PKC inhibitors. We used 1 and 10 μ M chelerythrine as an inhibitor of PKC because of its relative specificity for the enzyme (Herbert *et al.*, 1990). In some experiments, cells were cultured with 0.01 or 0.1 μ M staurosporine (Zilberman *et al.*, 1992) or 1, 5, or 10 μ M H-7 (Pugazhenthii *et al.*, 1990) to inhibit PKC. All three inhibitors have been shown to cause dose-dependent decreases in PKC activity of growth zone cell cultures (Sylvia *et al.*, 1998). [3 H]-Thymidine incorporation, [35 S]-sulfate incorporation, and alkaline phosphatase were measured as described above.

E. PGE₂ Production

Because PGE₂ is a regulator of growth zone chondrocytes (Schwartz *et al.*, 1999) and is involved in the rapid activation of PKC in these cells in response to other mediators (Schwartz *et al.*, 1998), we examined whether TGF- β 1 increases PGE₂ production as well. PGE₂ production by growth zone chondrocyte cultures treated with TGF- β 1 was assessed by using a radioimmunoassay kit (NEN DuPont, Boston, MA) as described previously (Schwartz *et al.*, 1992). PGE₂ has been shown to regulate physiologic responses of growth zone chondrocytes by signaling through the EP1 receptor (Sylvia *et al.*, 2001), which signals through the Gq/PLC pathway to activate PKC (Negishi *et al.*, 1995). To clarify whether the effect of TGF- β 1 on

PGE₂ production was before or after G-protein activation or PKC activation, confluent cultures were treated for 24 hours with 0.22 ng/ml TGF- β 1 in the presence or absence of 1, 10 or 100 μ M GTP γ S (general G-protein activator) or chelerythrine (PKC inhibitor).

F. Role of Protein Kinase A

PGE₂ exerts its effects on growth zone cells via the EP1 receptor through both PKC- and PKA-dependent mechanisms (Sylvia *et al.*, 2001). To assess the role of PKA in cell response to TGF- β 1, growth zone chondrocytes were incubated with TGF- β 1 \pm 1, 5, or 10 μ M H-8 (Pugazhenthii *et al.*, 1990). [³H]-Thymidine incorporation was examined in cultures treated with 0.88 ng/ml TGF- β 1, and alkaline phosphatase specific activity was measured in cultures treated with 0.22 ng/ml TGF- β 1 based on prior studies indicating that maximal effects of TGF- β 1 on these parameters occur at these concentrations (Schwartz *et al.*, 1993; Schwartz *et al.*, 1998). Assays were performed as described above.

G. TGF- β 1 Dependent Regulation of PKC

Chondrocyte culture lysates containing equivalent amounts of protein were mixed for 20 minutes with a lipid preparation containing phorbol-12-myristate-13-acetate, phosphatidylserine and Triton X-100 mixed micelles which provided the necessary cofactors and conditions for optimal activity (Bell *et al.*, 1986). To this mixture, a high affinity myelin basic protein peptide and ³²P-ATP (25 μ Ci/ml) was

added to a final assay volume of 50 μ l. After a 10-minute incubation in a 30°C waterbath, samples were spotted onto phosphocellulose discs and then washed twice with 1% phosphoric acid and once with distilled water to remove unincorporated label prior to placement in a scintillation counter.

1. Effect of Arachidonic Acid, Prostaglandins, and PGE₂

To assess the role of arachidonic acid in TGF- β 1-dependent PKC stimulation, confluent cultures were treated for 12 hours with vehicle alone or with 0.22 ng/ml TGF- β 1 in the presence or absence of 1, 10 or 100 μ M arachidonic acid. Control cultures were treated with TGF- β 1 + the arachidonic acid vehicle (0.02% ethanol in DMEM). To determine if prostaglandin mediates the effects of TGF- β 1 on PKC, growth zone chondrocytes were incubated in the presence or absence of 0.1, 1, or 10 μ M indomethacin \pm 0.22 ng/ml TGF- β 1 for 12 hours. Indomethacin blocks both Cox-1 and Cox-2 (Vane, 1971). The relative contributions of Cox-1 and Cox-2 were determined using resveratrol (0.1, 1, and 10 μ M) and NS-398 (0.1, 1, and 10 μ M) to inhibit the constitutive and inducible forms of the enzyme, respectively. The specific role of PGE₂ was determined by treating the cultures with vehicle \pm 0.22 ng/ml TGF- β 1 \pm 15, 60, or 240 pg/ml PGE₂.

2. Effect of Diacylglycerol

Diacylglycerol (DAG) activates PKC in a number of cells, including growth zone chondrocytes (Sylvia *et al.*, 1998). This is generally a rapid effect due to rapid Gq-dependent activation of PLC (Sylvia *et al.*, 1998). DAG then binds to PKC, activating translocation of PKC to the plasma membrane (Lapetina *et al.*, 1985).

Previous studies have shown that PLC was not involved in the mechanism of TGF- β 1-dependent PKC activation (Sylvia *et al.*, 1994). Thus, if DAG was involved, it must be generated through another pathway. DAG can also be produced through phospholipase D (PLD), but this pathway may not occur with the same rapidity as via PLC. To determine if diacylglycerol is involved in TGF- β 1 action, I examined the effects of dioctanoylglycerol (DOG), a cell-permeable form of the PKC activator (Lapetina *et al.*, 1985), and the diacylglycerol kinase inhibitor R59022, which maintains elevated diacylglycerol levels. R59022 blocks the conversion of diacylglycerol species to phosphatidic acid and is relatively promiscuous about the source of the diacylglycerol (de Chaffey de Courcelles *et al.*, 1985). We did not use butan-1-ol to block PLD (Nakamura *et al.*, 1997), because butanol is toxic to the cells. Cultures were treated with vehicle \pm 0.11 or 0.22 ng/ml TGF- β 1 in the presence or absence of 1, 10, or 100 μ M DOG or 5, 10 or 50 μ M R59022. Control cultures were treated with TGF- β 1 \pm vehicle (0.02% ethanol in DMEM). PKC activity was measured at 12 hours.

3. Direct Effect of TGF- β 1 on Membrane-associated PKC

PKC activity of membranes increases in response to a stimulus due to translocation of the activated cytosolic enzyme to the membrane (Newton, 1995). However, previous studies have shown that TGF- β 1-dependent increases in PKC are not due to translocation (Sylvia *et al.*, 1994). To determine whether TGF- β 1 regulates PKC activity by direct action on membrane-associated enzyme, isolated plasma membranes and matrix vesicles were incubated directly with TGF- β 1. Both

membrane fractions have been shown previously to contain PKC α and PKC ζ (Sylvia *et al.*, 1996); PKC α predominates in plasma membranes, whereas PKC ζ predominates in matrix vesicles. Because matrix vesicles are extracellular, they are right side out when they are isolated. They do not contain RNA or DNA, so new gene expression or protein synthesis cannot occur. Therefore, any effect of TGF- β 1 on PKC is by direct action on the membrane.

For these experiments, confluent, fourth passage growth zone cells were released from their matrix by trypsin digestion and collected by centrifugation. Plasma membranes were isolated by differential and sucrose density gradient centrifugation of homogenized cells (Schwartz *et al.*, 1988). Matrix vesicles were isolated by differential centrifugation of the trypsin-digested matrix (Ali *et al.*, 1970). Following assay for protein content (Smith *et al.*, 1985), plasma membranes or matrix vesicles were suspended in 0.9% NaCl and frozen at -70° C. Matrix vesicles isolated in this manner typically exhibit greater than two-fold enrichment of alkaline phosphatase specific activity when compared with the plasma membranes and have a transmission electron microscopic appearance consistent with matrix vesicles *in vivo* (Ali *et al.*, 1970). Matrix vesicles or plasma membranes (10 μ g protein in 0.9% NaCl containing 10% FBS) were incubated in the absence (vehicle only) or presence of a final concentration of 0.11, 0.22, or 0.88 ng/ml TGF- β 1 for either 9, 90, or 270 minutes at 37°C as described previously (Sylvia *et al.*, 1996). Following incubation, samples were assayed for PKC activity.

H. Statistical Management of the Data

Unless otherwise noted, the data presented here are from one of three or more independent experiments, all showing comparable results. Each data point represents the mean \pm SEM for six cultures (N=6). For membrane PKC assays, each data point is the mean \pm SEM for six membrane preparations, where each preparation was derived from one T-150 flask of cells. Data were analyzed by ANOVA. Statistical significance was determined using Bonferroni's modification of Student's t-test, with $P < 0.05$ being considered significant.

III. RESULTS

A. Role of TGF- β 1 Receptors

TGF- β 1 mediates its effects on growth zone chondrocytes through its type II surface receptor. Figure 1 shows how confluent growth zone cells were treated with control media or TGF- β 1 (0.22 ng/ml) in the absence or presence of TGF- β 1 type II receptor antibody. Antibodies to the TGF- β 1 type II receptor caused a statistically significant ($P < 0.05$) dose-dependent decrease in protein kinase C specific activity with a complete inhibitory effect at a maximum concentration of 2 μ g/ml. Figure 2 shows the effect of confluent growth zone cells treated with control media or TGF- β 1 in the absence or presence of TGF- β 1 type II receptor antibody. In this experiment [35 S]-sulfate incorporation decreased in a TGF- β 1 type II receptor antibody dose-dependent manner. The effect of TGF- β 1 type II receptor on TGF- β 1-induced [3 H]-thymidine incorporation is presented on Figures 3. Once again, antibodies to the TGF- β 1 type II receptor caused a dose-dependent decrease in [3 H]-thymidine incorporation. Figure 4 illustrates the effect of TGF- β 1 type II receptor on TGF- β 1-induced alkaline phosphatase specific activity. Confluent cells were treated with control media or TGF- β 1 in the presence or absence of TGF- β 1 type II receptor antibody. TGF- β 1 type II receptor antibody at a concentration of 2 μ g/ml caused a complete inhibitory effect on alkaline phosphatase specific activity. Figure 5 shows how the inhibitory effect of the TGF- β 1 type II soluble receptor was only partial with respect to protein kinase C activity even at concentration as high as 100 μ g/ml of soluble receptor. Nevertheless, the effect of TGF- β 1 type II soluble receptor on TGF- β 1-induced protein kinase C activity was statistically significant ($P < 0.05$). Similarly, the effect of TGF- β 1 type II soluble receptor on TGF- β 1-induced [35 S]-sulfate incorporation (Figure 6) shows a partial inhibitory effect. The effect of TGF- β 1 type II soluble receptor on TGF- β 1-induced [3 H]-thymidine

incorporation is presented on Figures 7. TGF- β 1 type II soluble receptor caused a dose-dependent decrease in [3 H]-thymidine incorporation at a concentration of 100 μ g/ml ($P < 0.05$). Figure 8 shows the effect of confluent growth zone cells treated with control media or TGF- β 1 in the absence or presence of TGF- β 1 type II soluble receptor. In this experiment alkaline phosphatase decreased in a TGF- β 1 type II soluble receptor dose-dependent manner. In contrast to TGF- β receptor II antibody and soluble receptor, TGF- β receptor III antibody and soluble receptor had no significant effect on protein kinase C specific activity in either control cultures or cultures treated with 0.22 ng/ml TGF- β 1 (data not shown). These results demonstrated that the effect of TGF- β 1 is initiated by binding of TGF- β 1 to the type II receptor. This dose-dependent decrease was statistically significant ($P < 0.05$).

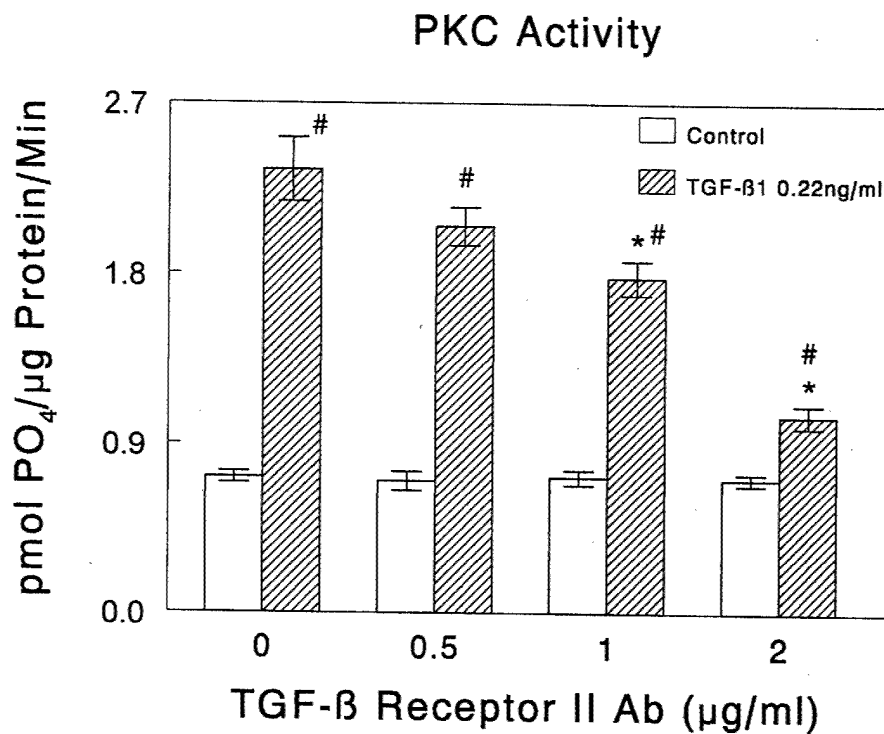


Figure 1: Effect of TGF-β1 type II receptor antibody on TGF-β1-induced protein kinase C (PKC) activity. Confluent growth zone cells were treated with control media or TGF-β1 in the absence or presence of antibody. Values are the mean \pm SEM of 6 cultures. *P < 0.05, vs. no treatment with antibody; #P < 0.05, vs. no treatment with TGF-β1.

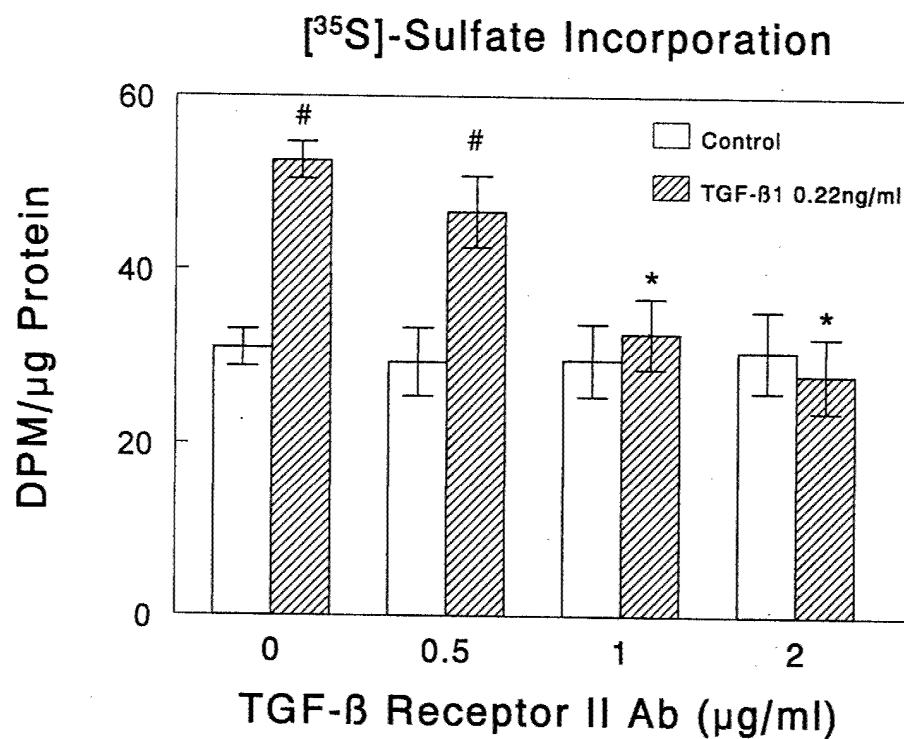


Figure 2: Effect of TGF-β1 type II receptor antibody on TGF-β1-induced [³⁵S]-sulfate incorporation. Confluent growth zone cells were treated with control media or TGF-β1 in the absence or presence of antibody. Values are the mean ± SEM of 6 cultures. *P < 0.05, vs. no treatment with antibody; #P < 0.05, vs. no treatment with TGF-β1.

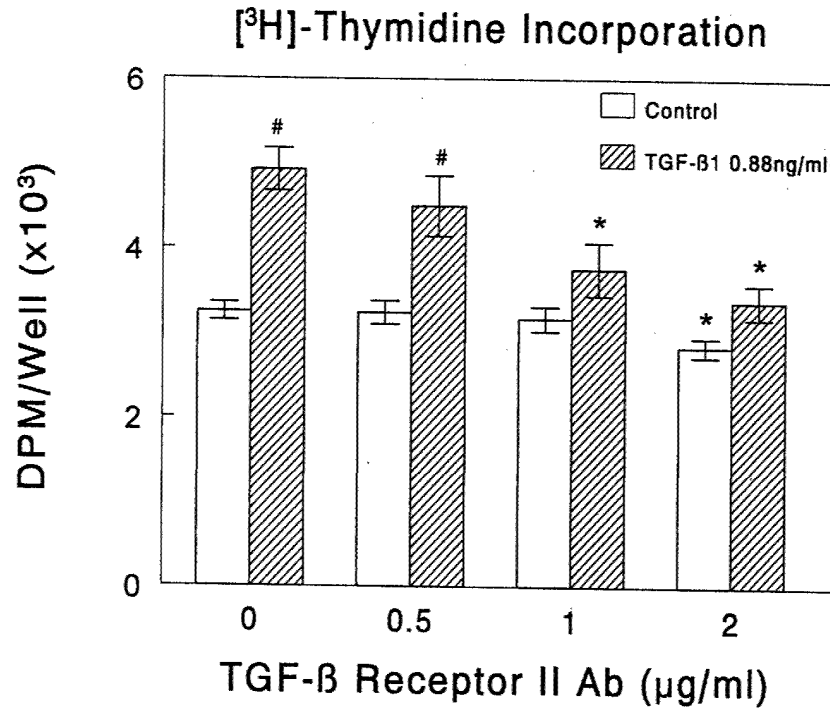


Figure 3: Effect of TGF-β1 type II receptor antibody on TGF-β1-induced [³H]-thymidine incorporation. Confluent growth zone cells were treated with control media or TGF-β1 in the absence or presence of antibody. Values are the mean ± SEM of 6 cultures. *P < 0.05, vs. no treatment with antibody; #P < 0.05, vs. no treatment with TGF-β1.

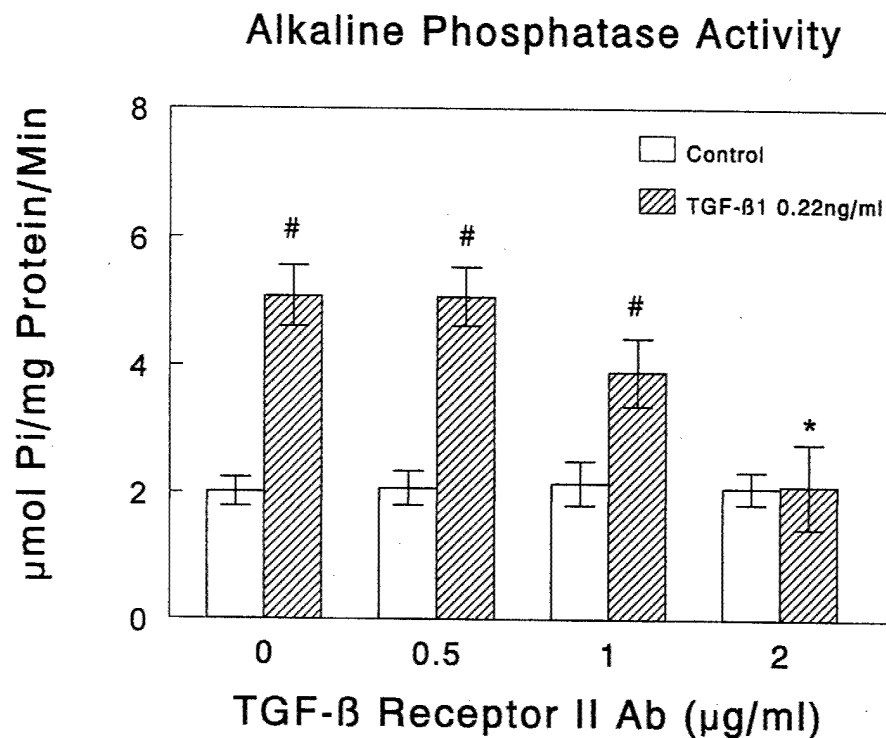


Figure 4: Effect of TGF- β 1 type II receptor antibody on TGF- β 1-induced alkaline phosphatase activity. Confluent growth zone cells were treated with control media or TGF- β 1 in the absence or presence of antibody. Values are the mean \pm SEM of 6 cultures. *P < 0.05, vs. no treatment with antibody; #P < 0.05, vs. no treatment with TGF- β 1.

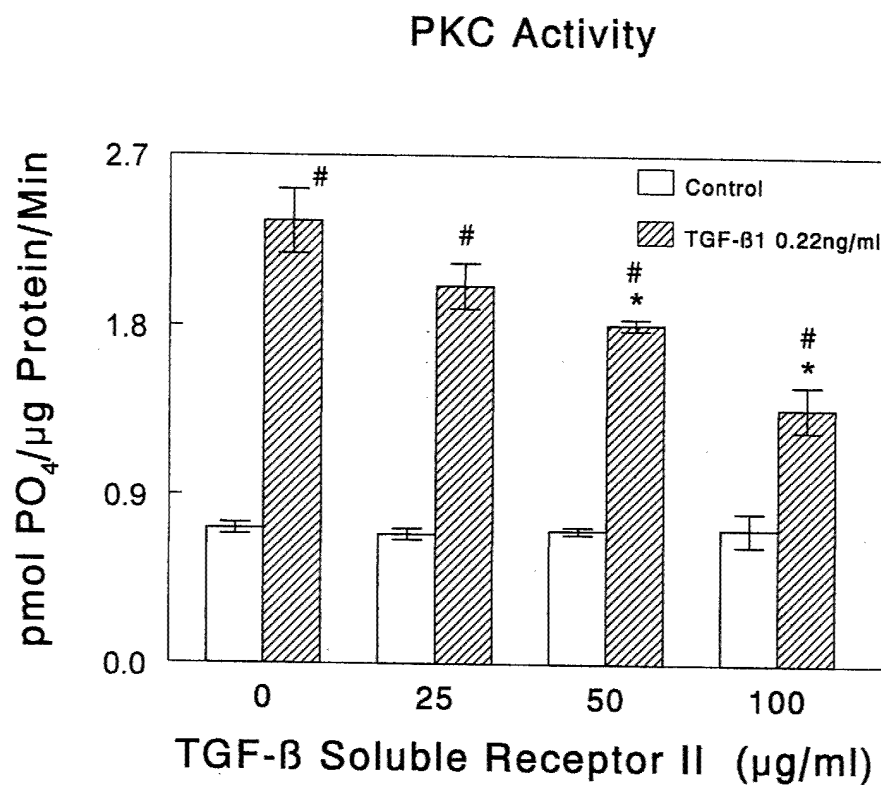


Figure 5: Effect of TGF-β1 soluble type II receptor on TGF-β1-induced protein kinase C (PKC) activity. Confluent growth zone cells were treated with control media or TGF-β1 in the absence or presence of antibody. Values are the mean \pm SEM of 6 cultures. *P < 0.05, vs. no treatment with antibody; #P < 0.05, vs. no treatment with TGF-β1.

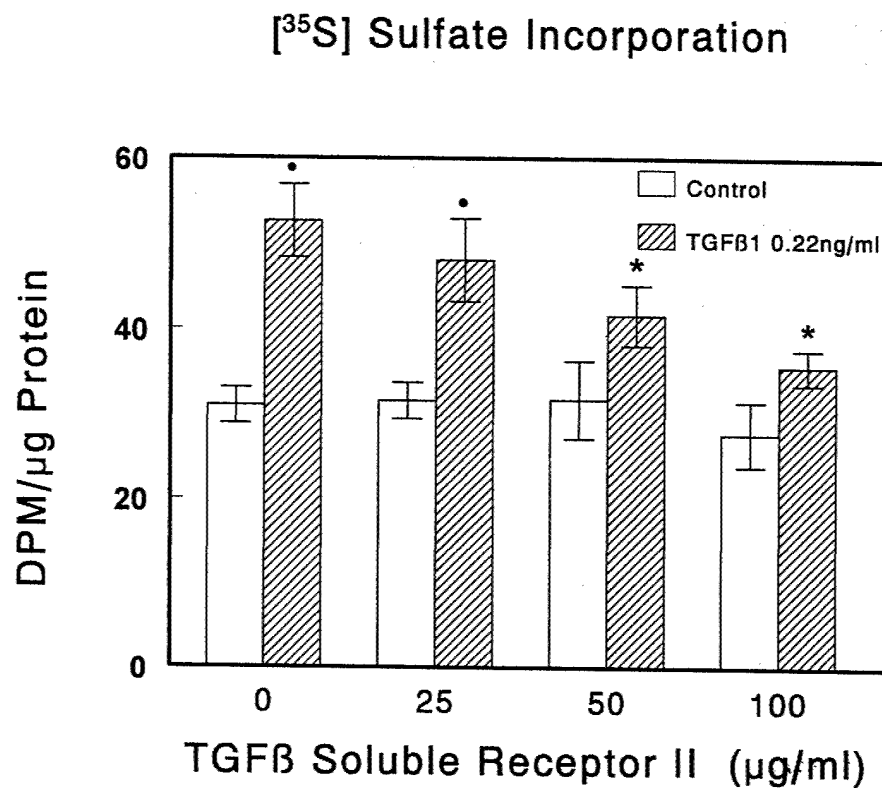


Figure 6: Effect of TGF-β1 soluble type II receptor on TGF-β1-induced [³⁵S]-sulfate incorporation. Confluent growth zone cells were treated with control media or TGF-β1 in the absence or presence of antibody. Values are the mean ± SEM of 6 cultures. *P < 0.05, vs. no treatment with antibody; #P < 0.05, vs. no treatment with TGF-β1.

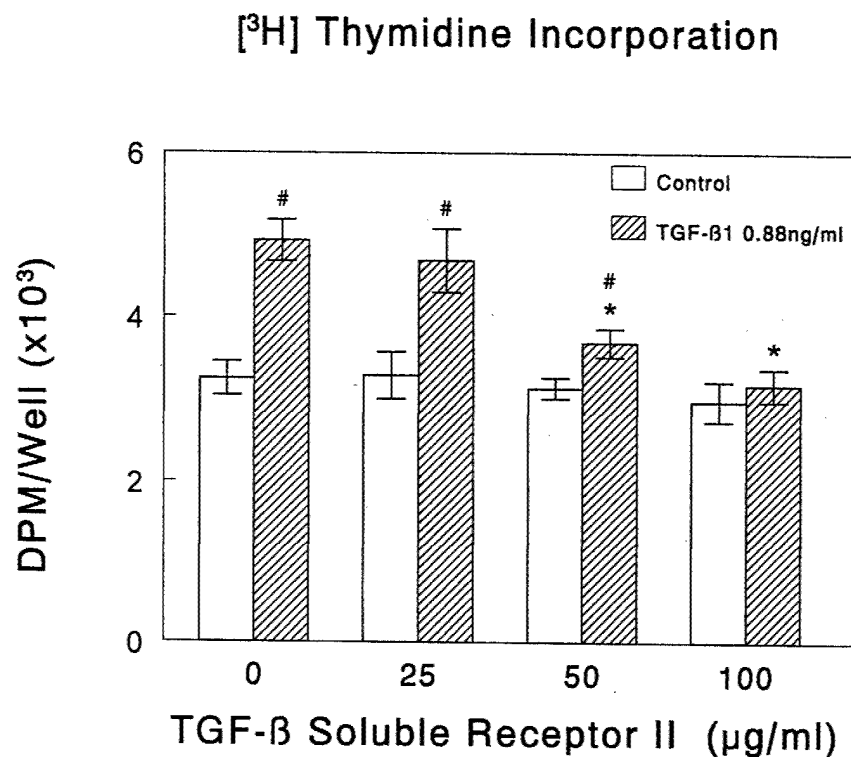


Figure 7: Effect of TGF- β 1 soluble type II receptor on TGF- β 1-induced [³H]-thymidine incorporation. Confluent growth zone cells were treated with control media or TGF- β 1 in the absence or presence of antibody. Values are the mean \pm SEM of 6 cultures. * $P < 0.05$, vs. no treatment with antibody; # $P < 0.05$, vs. no treatment with TGF- β 1.

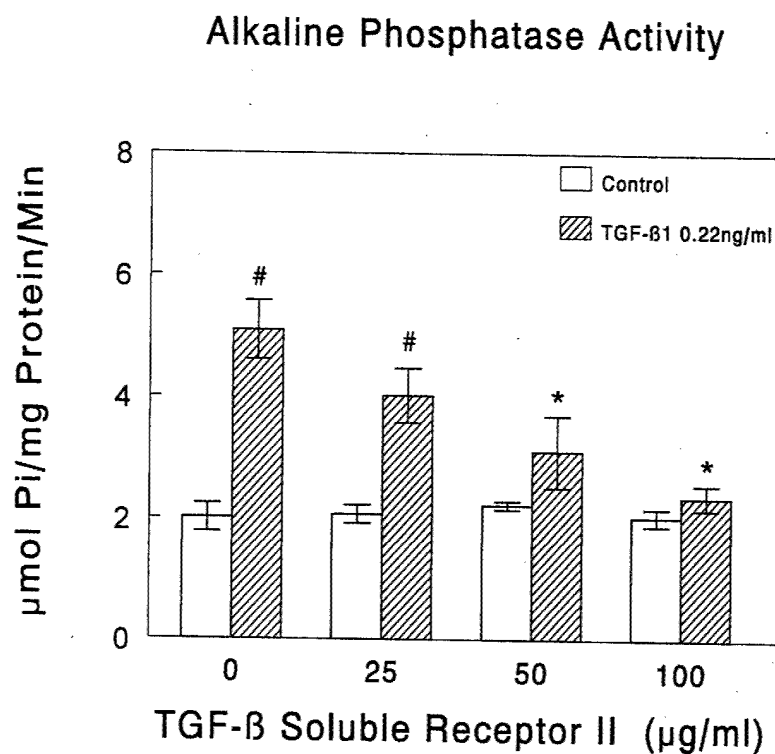


Figure 8: Effect of TGF- β 1 soluble type II receptor on TGF- β 1-induced alkaline phosphatase activity. Confluent growth zone cells were treated with control media or TGF- β 1 in the absence or presence of antibody. Values are the mean \pm SEM of 6 cultures. * $P < 0.05$, vs. no treatment with antibody; # $P < 0.05$, vs. no treatment with TGF- β 1.

B. Role of PKC

The growth zone chondrocytes response to TGF- β 1 was protein kinase C-dependent. Chelerythrine at different concentrations (1 μ M and 10 μ M) reduced the TGF- β 1-dependent increase on [3 H]-thymidine incorporation (Figure 9) as compared to the controls at TGF- β 1 concentration of 0 ng/ml, 0.11 ng/ml, 0.22 ng/ml, and 0.88 ng/ml. Figure 10A shows the effect of the protein kinase C inhibitor chelerythrine (1 μ M) on TGF- β 1-induced [35 S]-sulfate incorporation. Proteoglycan sulfation was significantly inhibited by chelerythrine in cultures treated with 0.11 ng/ml, 0.22 ng/ml, and 0.88 ng/ml of TGF- β 1. Similarly, chelerythrine (1 μ M) significantly ($P < 0.05$) inhibited alkaline phosphatase specific activity (Figure 10B). The Protein kinase C-dependent effects of TGF- β 1 on [3 H]-thymidine incorporation were confirmed using the protein kinase C inhibitor staurosporine (Figure 11). Staurosporine significantly inhibited TGF- β 1-induced [3 H]-thymidine incorporation at different concentrations (0.01 μ M, 0.1 μ M), but a complete inhibitory effect was observed at the higher concentration (0.1 μ M). Figure 12 shows the effect of Protein kinase C inhibitor H-7, and protein kinase A inhibitor H-8 on TGF- β 1-induced [3 H]-thymidine incorporation. The protein kinase C inhibitor H-7 significantly ($P < 0.05$) reduced the TGF- β 1-induced [3 H]-thymidine incorporation. The protein kinase A inhibitor H-8 partially reduced TGF- β 1-stimulated [3 H]-thymidine incorporation (Figure 12). This demonstrates that part of the response to TGF- β 1 was also mediated via a protein kinase A-dependent mechanism. H-8 also partially blocked the TGF- β 1-stimulated increase in alkaline phosphatase specific activity.

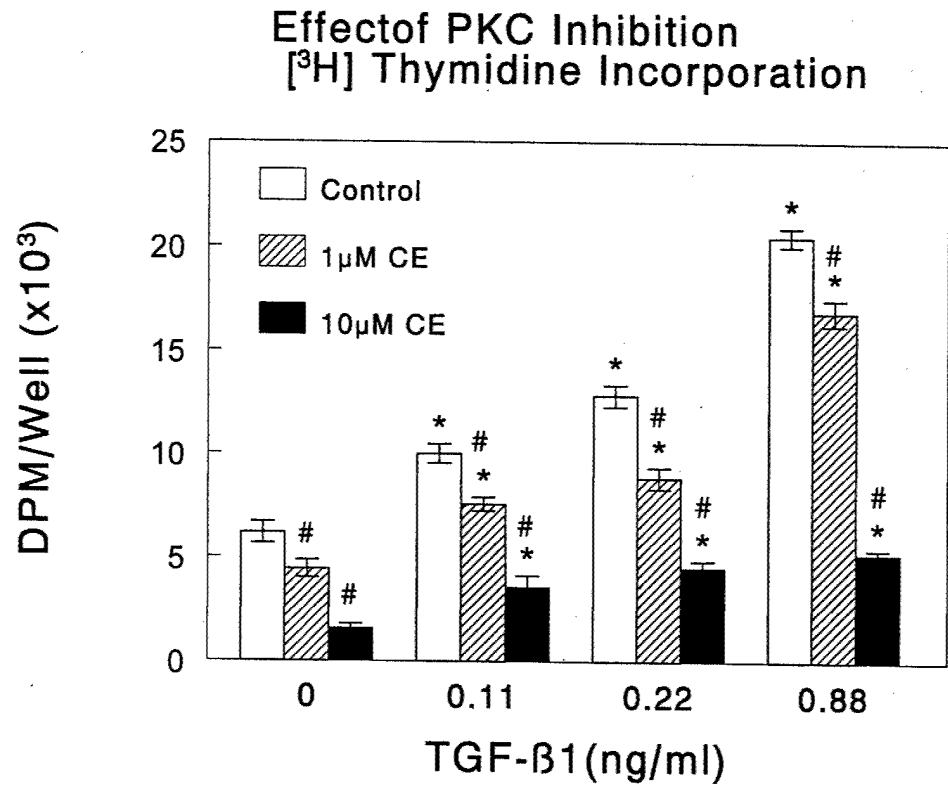


Figure 9: Effect of PKC inhibitor, chelerythrine, on TGF-β1-induced [³H]-thymidine incorporation. Values are the mean ± SEM of 6 cultures. *P<0.05, vs. no treatment with TGF-β1; #P<0.05, vs. no treatment with PKC inhibitor.

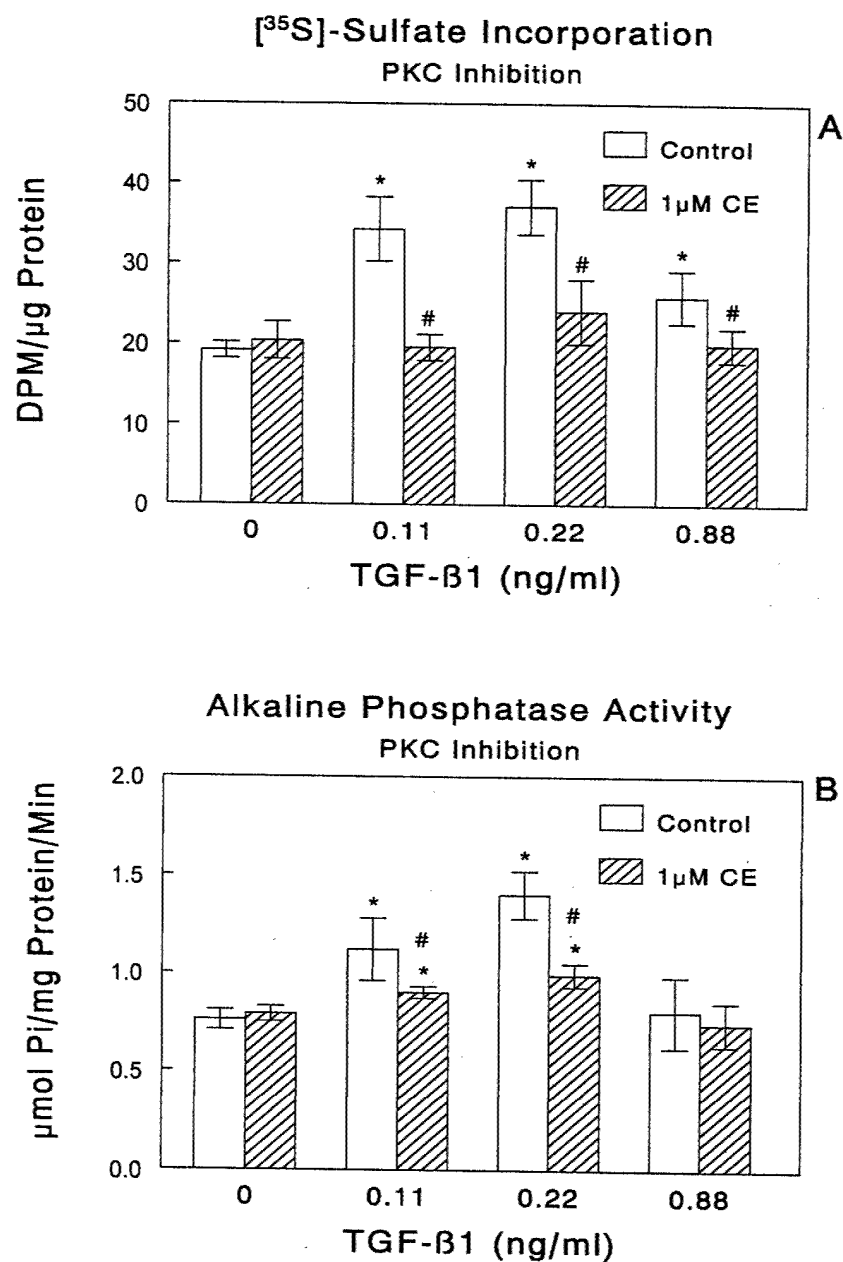


Figure 10. Effect of PKC inhibitor, chelerythrine, on TGF- β 1-induced [³⁵S]-sulfate incorporation (A); and alkaline phosphatase specific activity (B). Values are the mean \pm SEM of 6 cultures. *P<0.05, vs. no treatment with TGF- β 1; #P<0.05, vs. no treatment with PKC inhibitor.

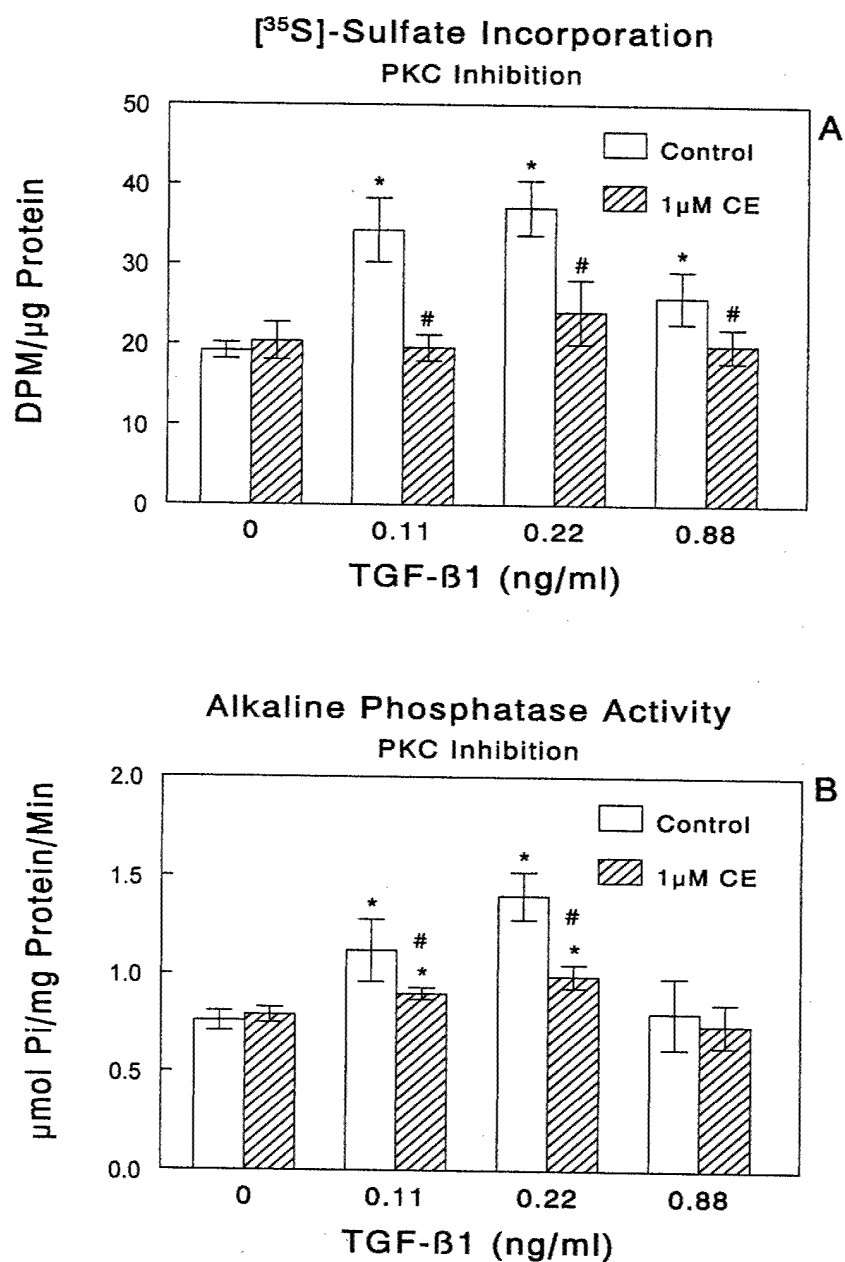


Figure 10. Effect of PKC inhibitor, chelerythrine, on TGF-β1-induced [³⁵S]-sulfate incorporation (A); and alkaline phosphatase specific activity (B). Values are the mean ± SEM of 6 cultures. *P<0.05, vs. no treatment with TGF-β1; #P<0.05, vs. no treatment with PKC inhibitor.

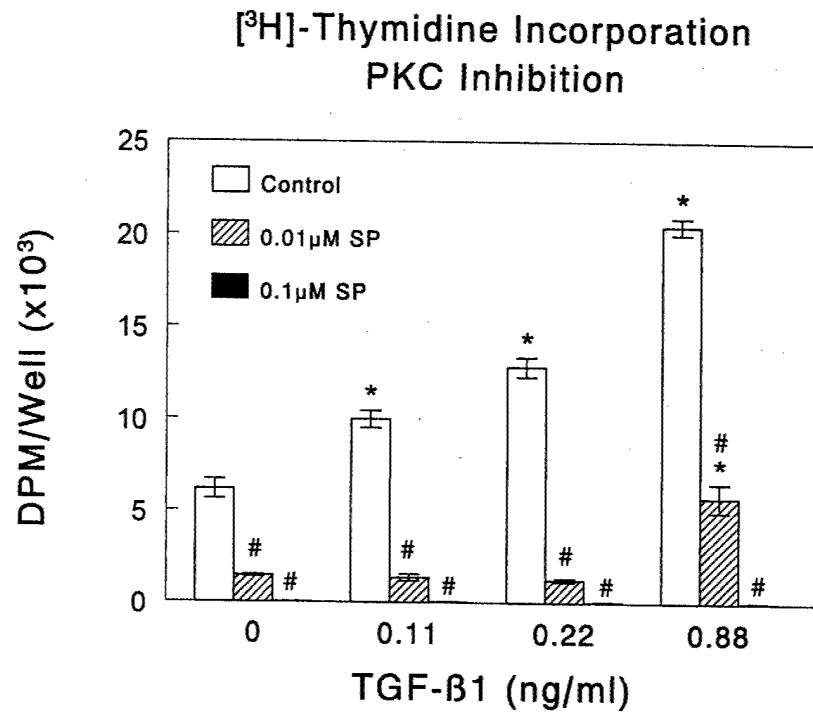


Figure 11. Effect of PKC inhibitor, staurosporine, on TGF-β1-induced [³H]-Thymidine incorporation. Values are the mean ± SEM of 6 cultures. *P<0.05, vs. no treatment with TGF-β1; #P<0.05, vs. no treatment with PKC inhibitor.

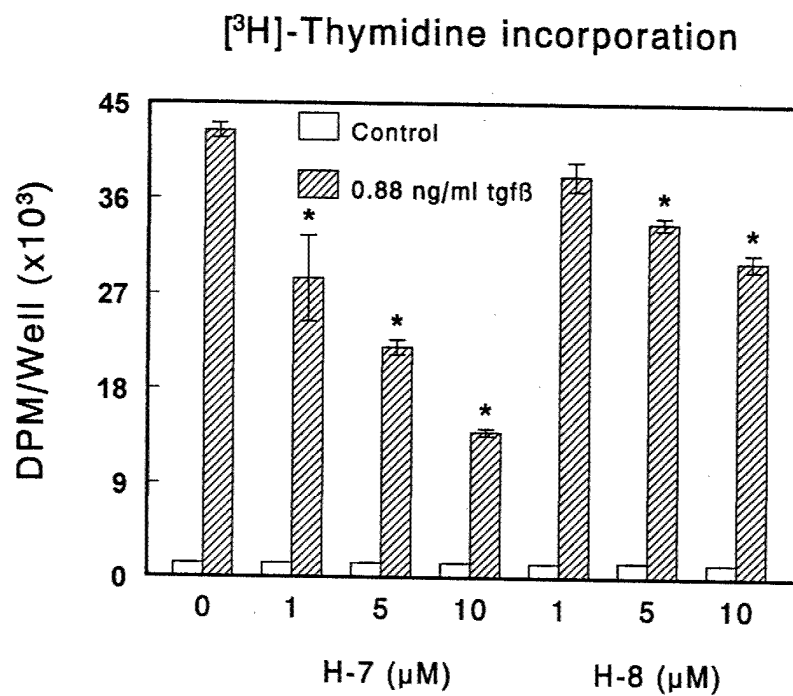


Figure 12. Effect of PKC/PKA inhibitors, H-7 and H-8, on TGF- β 1-induced [³H]-Thymidine incorporation. Values are the mean \pm SEM of 6 cultures. * $P < 0.05$, vs. no treatment with TGF- β 1; # $P < 0.05$, vs. no treatment with PKC/PKA inhibitor.

Figure 13A shows how the production of prostaglandin E₂ by growth zone chondrocyte cultures after 12 hours was increased by TGF- β 1 in a biphasic manner. Statistically significant increases ($P < 0.05$) in prostaglandin E₂ production were observed for cultures treated with 0.12, 0.22 and 0.5 ng/ml TGF- β 1, with the highest at 0.22 ng/ml. PGE₂ production varied with time of treatment (Figure 13B). In the control group, PGE₂ levels were increased by 12 hours. The treated group had TGF- β 1 in a concentration of 0.22 ng/ml, and this caused an increase in PGE₂ production when compared to the control group. The greatest increase was observed at the 12 hours point, but a statistically significant increase was maintained after 24 hours.

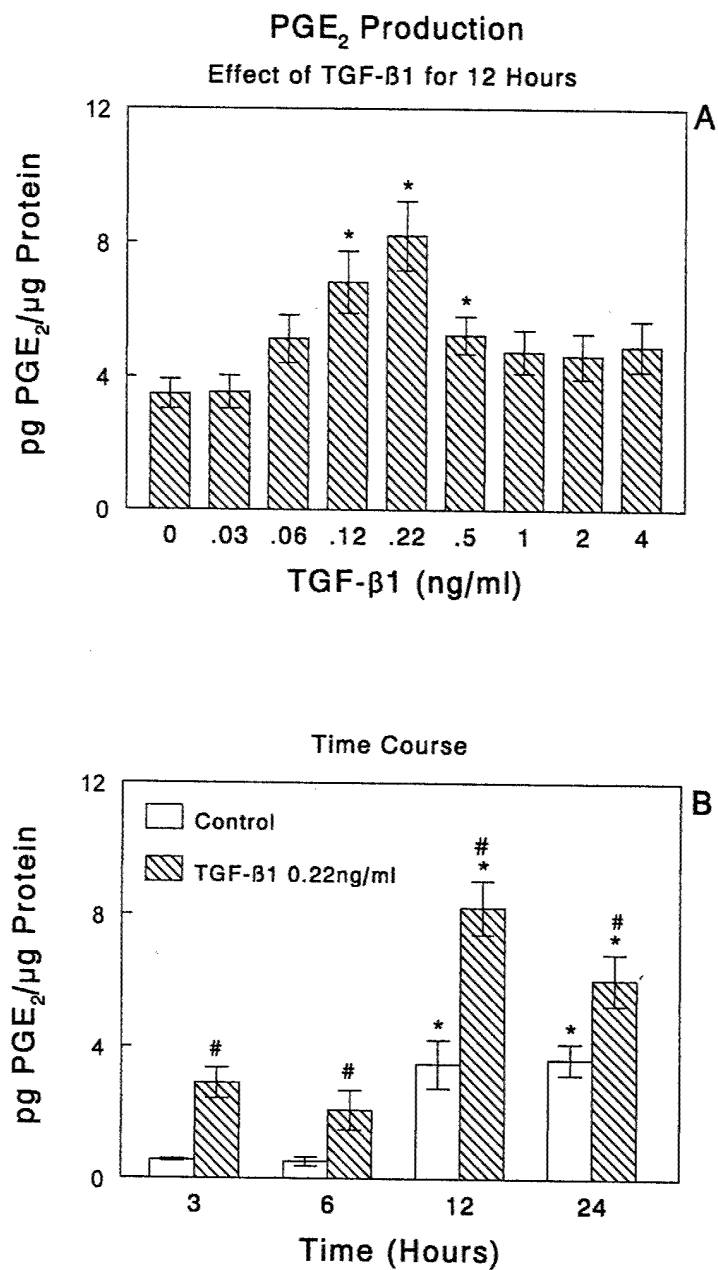


Figure 13. Effect of TGF-β1 on PGE₂ production. Dose-dependent response to TGF-β1 (A); time course (B). Values are the mean ± SEM of 6 cultures. *P<0.05, vs. control (A), 3 hrs (B), or no activator (C); #P<0.05, vs. not treated with TGF-β1.

The effect of TGF- β 1 on prostaglandin E₂ production was G-protein-dependent. Figure 14 shows how the G-protein activator GTP γ S had no effect on the prostaglandin E₂ production of the control group, but increased TGF- β 1-induced prostaglandin E₂ production in a dose-dependent manner. The maximal increases were observed in cultures treated with 10 μ M GTP γ S. In contrast, PGE₂ production in response to TGF- β 1 was not mediated by PKC. Inhibition of PKC with 1 μ M chelerythrine had no effect on PGE₂ production in either control cultures or cultures treated with TGF- β 1.

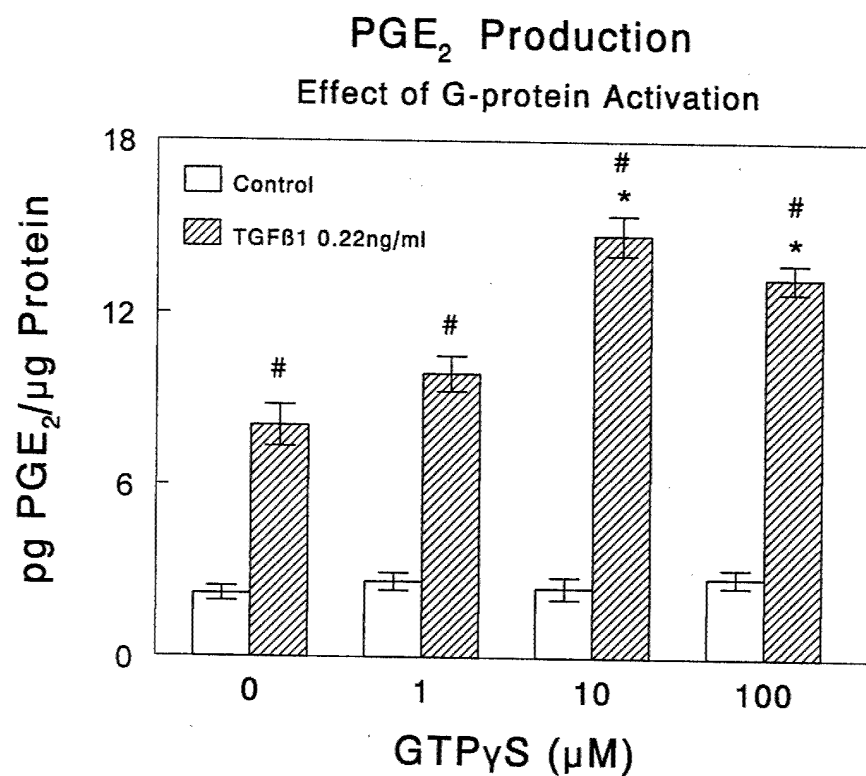


Figure 14. Effect of TGF-β1 on PGE₂ production. Dose-dependent response to G-protein activator GTPγS (C). Values are the mean ± SEM of 6 cultures. *P<0.05, vs. control (A), 3 hrs (B), or no activator (C); #P<0.05, vs. not treated with TGF-β1.

C. Mechanism of action of TGF- β 1

The inhibition of cyclooxygenase with indomethacin (a nonspecific cyclooxygenase inhibitor) reduced TGF- β 1-induced protein kinase C activity in a dose-dependent manner (Figure 15A). Protein kinase C activity in the control group and TGF- β 1-induced activity were reduced with increasing concentrations of indomethacin. Cyclooxygenase-1 was responsible for this effect, since the cyclooxygenase-1 inhibitor, resveratrol, significantly reduced protein kinase C activity at a concentration of 10 μ M (Figure 15B). The cyclooxygenase-2 inhibitor NS-398 had no effect on protein kinase C activity in either control or TGF- β 1-treated cultures (Figure 15C).

Effect of Cox Inhibition on PKC Activity

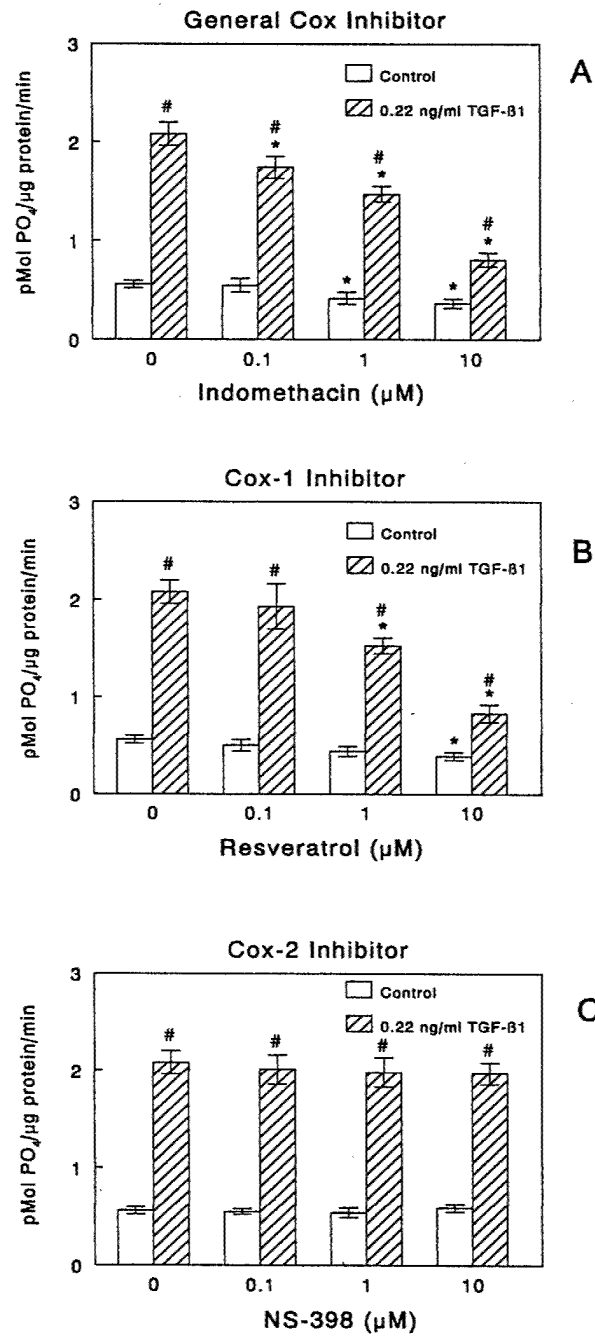


Figure 15. Effect of cyclooxygenase inhibitors on TGF- β 1-induced PKC activity. Growth zone cells were treated with control media or TGF- β 1 in the presence or absence of indomethacin (A), resveratrol (B), or NS-398 (C). Values are the mean \pm

SEM of 6 cultures. * $P < 0.05$, vs. no inhibitor; # $P < 0.05$, vs. not treated with TGF- $\beta 1$. G-proteins are involved in the action of TGF- $\beta 1$ in GC cells. The G-protein inhibitors pertussis toxin (Figure 16A), and cholera toxin (Figure 16B), which target G_i -proteins and G_s -proteins respectively, both increased protein kinase C activity slightly in the control cultures, but significantly ($P < 0.05$) reduced TGF- $\beta 1$ -induced protein kinase C activity in a dose-dependent manner. At a concentration of 100 ng/ml, both inhibitors completely blocked the stimulatory effect of TGF- $\beta 1$ on protein kinase C. The same results were observed using the general G-protein inhibitor GDP β S (Figure 17). At the highest concentration used (100 ng/ml), the effect of TGF- $\beta 1$ was completely blocked. When the G-protein activator GTP γ S was used a significant ($P < 0.05$) dose-dependent increase in protein kinase C activity was observed (Figure 18).

Effect of Inhibiting G_i or G_s Protein on PKC Activity

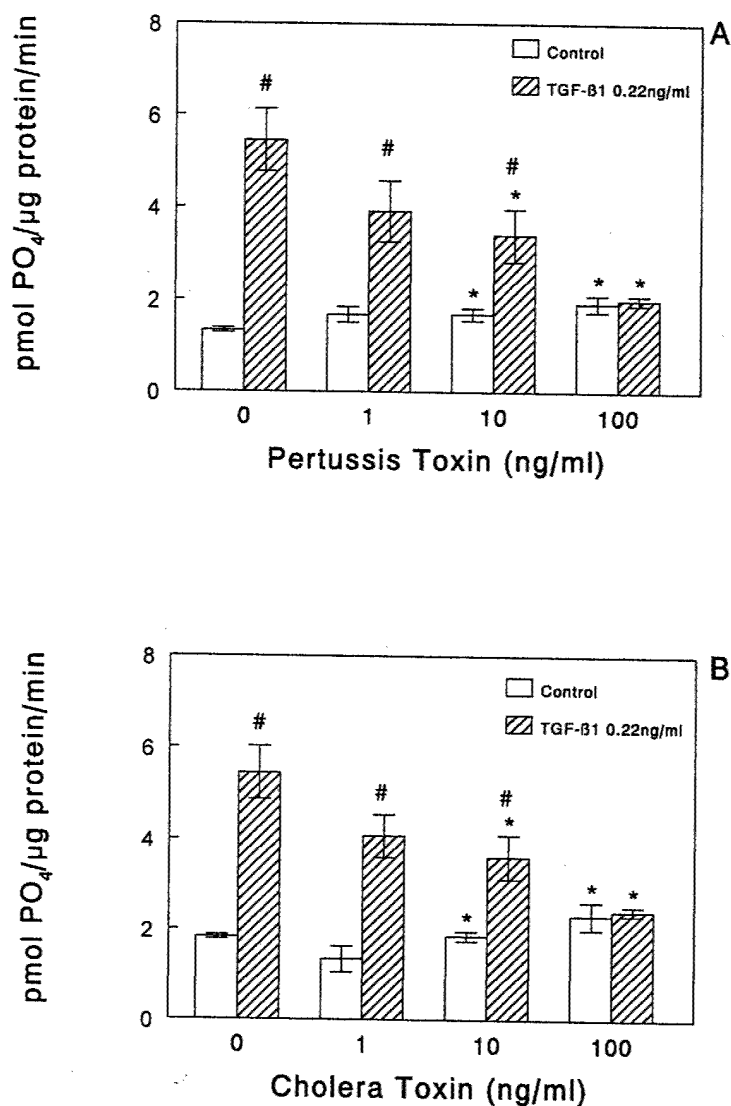


Figure 16. Role of G-proteins in TGF- $\beta 1$ -induced PKC activity. Growth zone cells were treated with control media or TGF- $\beta 1$ in the presence or absence of pertussis toxin (A), cholera toxin (B). Values are the mean \pm SEM of 6 cultures. * $P < 0.05$, vs. no inhibitor or activator; # $P < 0.05$, vs. not treated with TGF- $\beta 1$.

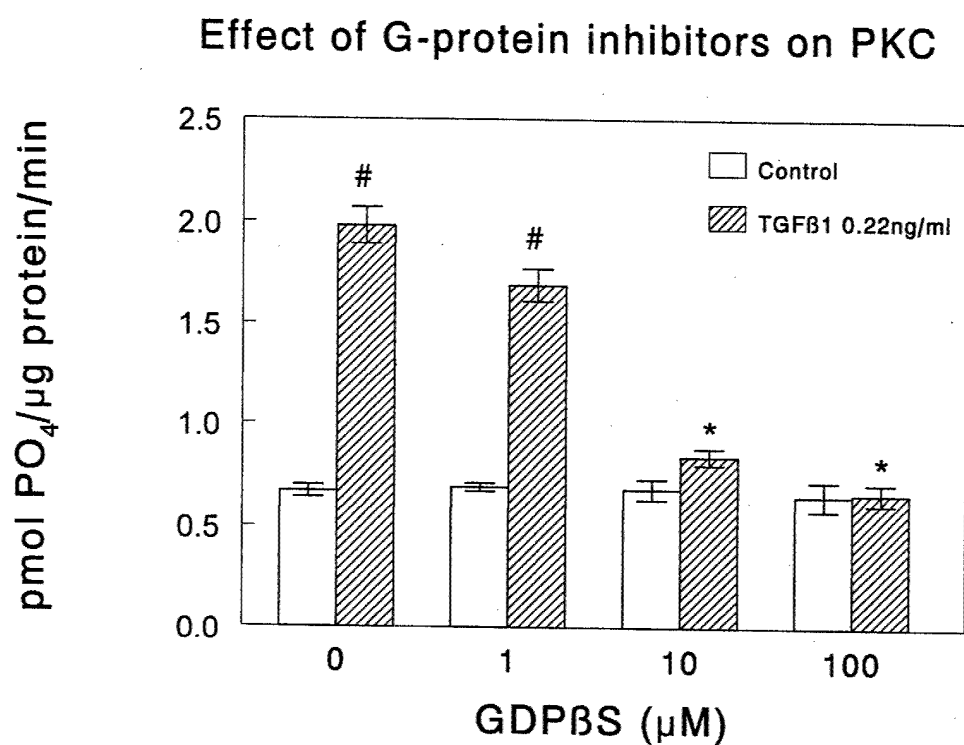


Figure 17. Role of G-proteins in TGF- β 1-induced PKC activity. Growth zone cells were treated with control media or TGF- β 1 in the presence or absence of G-protein inhibitor GDP β S. Values are the mean \pm SEM of 6 cultures. * $P < 0.05$, vs. no inhibitor or activator; # $P < 0.05$, vs. not treated with TGF- β 1.

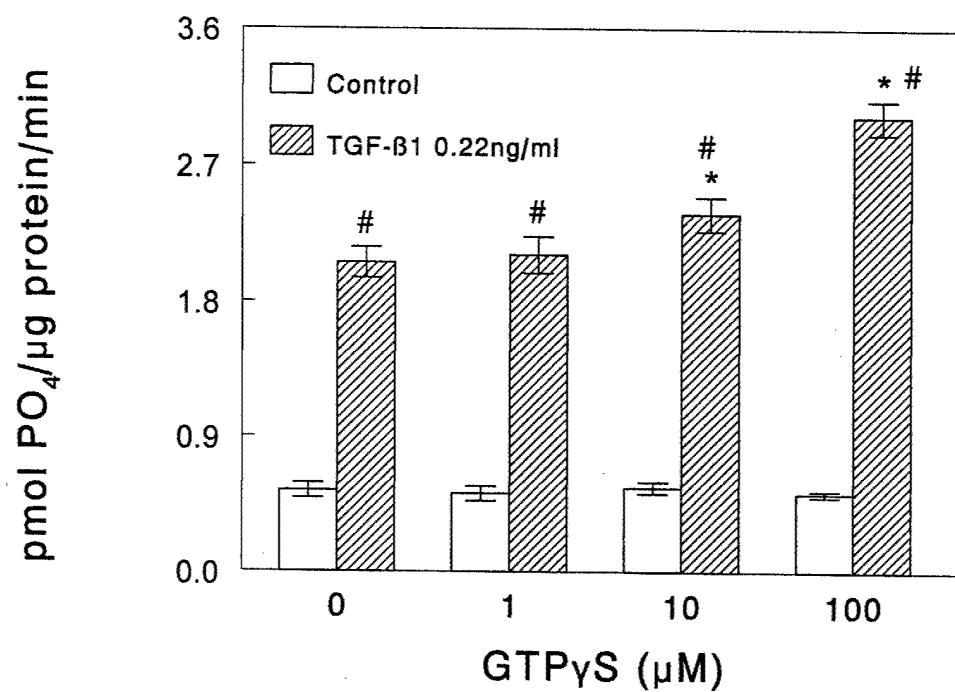


Figure 18. Role of G-proteins in TGF-β1-induced PKC activity. Growth zone cells were treated with control media or TGF-β1 in the presence or absence of G-protein activator GTPγS. Values are the mean ± SEM of 6 cultures. *P<0.05, vs. no inhibitor or activator; #P<0.05, vs. not treated with TGF-β1.

Protein kinase A is involved in TGF- β 1 regulation of protein kinase C activity in GC cells. The protein kinase A inhibitor H-8 caused a small, but significant, dose-dependent decrease in PKC in the control cultures, and a larger dose-dependent decrease ($P < 0.05$) on TGF- β 1-stimulated effect in PKC activity (Figure 19).

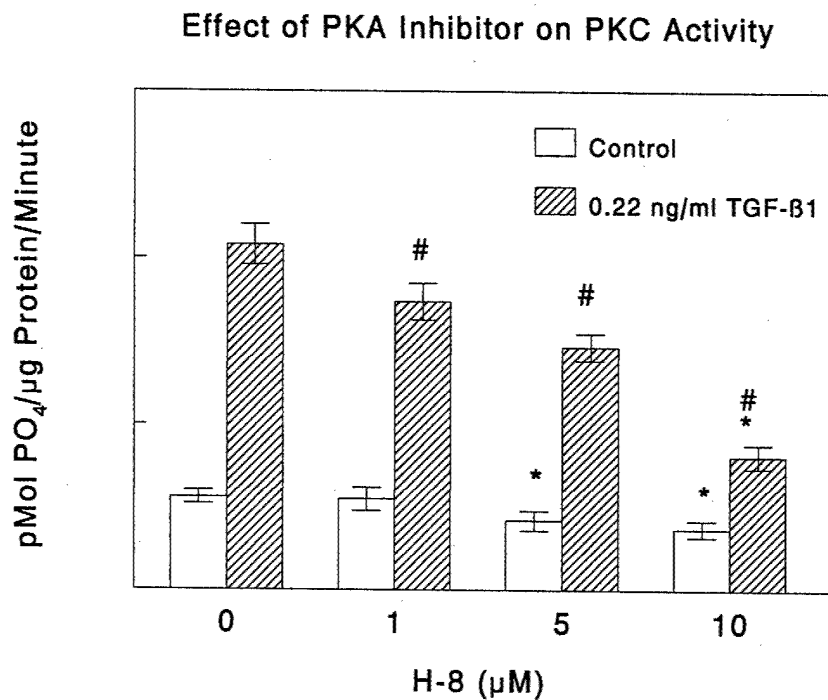


Figure 19: Effect of PKA inhibitor on PKC activity in growth zone chondrocytes. Confluent growth zone cells were treated with control media or TGF-β1 in the presence or absence of the PKA inhibitor H-8. Values are the mean \pm SEM of 6 cultures. #P<0.05, vs. no treatment with TGF-β1; *P<0.05, vs. no treatment with H-8.

IV. DISCUSSION

The purpose of this study was to determine if the effect of TGF- β 1 on growth zone chondrocytes is mediated through TGF- β receptor II or III, and to determine the signal transduction pathways involved. Previous studies have shown the involvement of Smad proteins as intracellular mediators of TGF- β signal transduction. This study demonstrates that TGF- β 1 mediates its effects on proliferation, proteoglycan production, and alkaline phosphatase activity of costochondral growth zone chondrocytes by a mechanism involving TGF- β surface receptor II, and not the TGF- β surface receptor III. The involvement of TGF- β receptor II in the signal transduction of TGF- β 1 has been demonstrated by others. The addition of TGF- β 1 receptor II antibodies and TGF- β soluble receptors blocked the effects of the growth factor on cell proliferation, alkaline phosphatase activity, and proteoglycan production. The type III receptor does not appear to be involved, since antibodies to this receptor and addition of soluble receptor had no effect.

The effects of TGF- β 1 on the parameters examined here are initiated by binding of TGF- β 1 to the type II receptor, and mediated by PKC. The inhibition of PKC blocks the stimulatory effects of TGF- β 1 on cell proliferation, alkaline phosphatase activity, and proteoglycan production. However, not all of the physiological responses of growth zone cells to TGF- β 1 are mediated by PKC. PKA also plays a role, since inhibition of this signaling pathway caused a partial reduction in the stimulatory effect of TGF- β 1 on proliferation and alkaline phosphatase activity. PKA may mediate the response of growth zone cells to TGF- β 1 directly or indirectly through modulation of PKC, based on the decrease in TGF- β 1-stimulated PKC activity in cultures treated with the PKA inhibitor H-8. TGF- β 1 also caused an increase in PGE₂ production that was dependent on G-proteins.

This study used selective inhibitors to specifically discriminate between the contributions of the PKC and PKA signaling pathways in the response of growth plate chondrocytes to TGF- β 1. Signaling pathway studies like this one are complicated by the overlapping sensitivities of these enzymes to the inhibitors used, and for that reason we used concentrations that were optimized for the specific pathway studied. For example, chelerythrine has an IC_{50} of 0.7 μ M for PKC, whereas its IC_{50} for PKA is 170 μ M (Herbert *et al.*, 1990); the highest concentration of chelerythrine used here was 1 μ M. Moreover, the contribution of the PKC signaling pathway to the physiological response was verified using alternate inhibitors, again taking care to use doses optimized for PKC. H-7 has been used effectively in other systems to block PKC activity in the same dose range as used in this study (Quick *et al.*, 1992). H-7 also inhibits other kinases including PKA, PKB, and myosin light chain kinase (MLCK) (Quick *et al.*, 1992), but the concentrations of H-7 required to do so are considerably greater than the highest dose used in the present study. Similarly, staurosporine can inhibit other serine/threonine kinases as well as some tyrosine kinases. The doses used in this study are appropriate for PKC, since staurosporine inhibits PKC with an IC_{50} of 0.7 nM and inhibits PKG with an IC_{50} of 85 nM (Yanigihara *et al.*, 1991). Inhibition of tyrosine kinases requires 25-50 μ M. A study by Osaki *et al.*, 1999 using rat articular chondrocytes support the involvement of PKA and PKC. When the articular chondrocytes were treated with 50 μ M H-7, the stimulatory effect of TGF- β 1 on DNA synthesis was blocked. The levels of H-7 used exceed the IC_{50} for PKC (3.0 μ M), thereby demonstrating the involvement of both kinases.

The present study demonstrates that diacylglycerol is not involved in the TGF- β 1-dependent activation of PKC, since exogenous diacylglycerol had no effect on TGF- β 1-stimulated PKC. Also, increasing endogenous diacylglycerol by inhibiting

DAG kinase failed to affect PKC in response to TGF- β 1. In general, diacylglycerol is produced as a rapid response to receptor activation, but TGF- β 1-dependent PKC is a delayed response, implicating a regulatory cascade. The results indicate that no component of the cascade mediates its effects via PLC or diacylglycerol, since DOG or the DAG kinase inhibitor were present throughout the 12-hour incubation. This supports previous observations by Sylvia *et al.*, 1994 showing that the stimulatory effect of TGF- β 1 on PKC activity does not involve PLC, based on the inhibition of PLC activity with the PI-PLC inhibitor U73122, and it also rules out the possibility of a role for diacylglycerol produced through a PLD dependent mechanism.

In the present study we concluded that, as noted previously for resting zone cells (Schwartz *et al.*, 1998), G-proteins also mediate the effects of TGF- β 1 on PKC in growth zone chondrocytes. When we added the G-protein inhibitor GDP β S, PKC activity in response to TGF- β 1 was blocked. Both G_i and G_s appear to be involved, since TGF- β 1 stimulation of PKC can be blocked by pertussis toxin, which is an specific G_i inhibitor, and cholera toxin, which is an specific G_s inhibitor. GDP γ S, a G-protein activator, increased TGF- β 1-dependent PKC activity.

The effect of TGF- β 1 on PKC in growth zone cells is also mediated by the PLA₂ pathway. Arachidonic acid, the product of PLA₂, is the substrate for cyclooxygenase, which results in prostaglandin production. TGF- β 1 stimulated maximal PGE₂ release at a concentration of 0.22 ng/ml and at 12 hours, a time that resulted in maximal increases in PKC activity. This suggests that PGE₂ might mediate the effect of TGF- β 1 on PKC. The increase in PGE₂ levels is not due to an increased rate of metabolism of arachidonic acid because only inhibition of Cox-1 blocked the TGF- β 1-dependent effect. Inhibition by indomethacin, a non-specific Cox inhibitor, and inhibition of Cox-1 using resveratrol, resulted in decreased PKC activity. In contrast, inhibition of Cox-2 had no effect on TGF- β 1-dependent PKC

activity. Also, prostaglandins exert their effects on cells via EP receptors, which are G-protein-coupled, potentially accounting for the inhibition in PKC activity when G-proteins are inhibited. This pathway may contribute to PKC activation in response to TGF- β 1, as suggested by the inhibition of PKA with H-8 causing a reduction in TGF- β 1-induced PKC activity.

The results of this study show that the effects of TGF- β 1 on growth zone chondrocytes are mediated by the type II receptor involving at least three separate pathways: PKC, PLA₂/arachidonic acid/prostaglandin, and PKA. Also, we can conclude that these pathways are interdependent. Figure 20 presents a diagram with the proposed pathways of TGF- β 1 signal transduction. First, TGF- β 1 binds to its type II receptor in the cell membrane, which phosphorylates and binds to the type I receptor. TGF- β 1 type I receptor activates Smad proteins, but also activates a G-protein, altering the activity of PLA₂ by phosphorylation, which produces arachidonic acid. Arachidonic acid is metabolized by cyclooxygenase to prostaglandins, which bind to EP1 and EP2 receptors on the growth zone chondrocyte cell surface (Sylvia *et al*, 2001). EP1 signaling stimulates PKC activity, possibly through a G-protein, while EP2 signaling increases cAMP, which activates PKA. This suggests that TGF- β 1 signals through PKC and PKA to increase alkaline phosphatase activity and to stimulate proliferation and through PKC to stimulate proteoglycan production.

Therefore, I conclude that TGF- β 1 regulates growth plate chondrocytes by a mechanism that is initiated through the TGF- β 1 type II receptor, and involves PKC, PKA, and PLA₂/arachidonic acid/prostaglandin pathways. These pathways are essential for the proliferation and differentiation of these cells.

Effect of TGF- β 1 on Growth Zone Chondrocytes

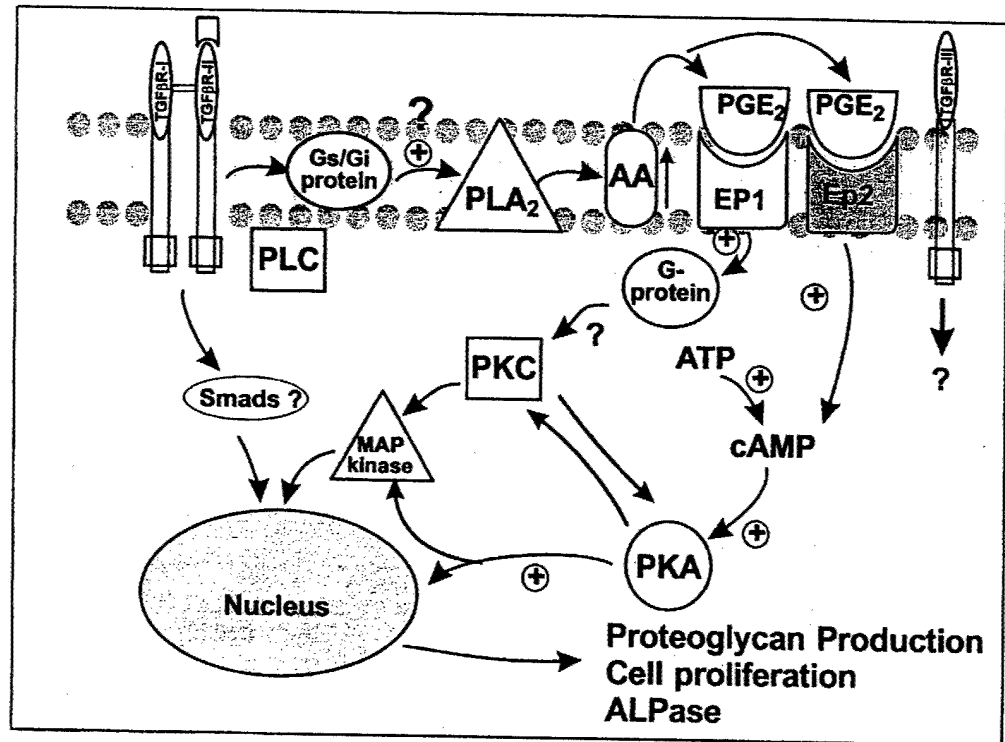


Figure 20. Proposed pathways of TGF- β 1 regulation of growth plate chondrocytes. AA: arachidonic acid, ALPase: alkaline phosphatase, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, PKA: protein kinase A, PKC; protein kinase C, PLA; phospholipase A, PLC: phospholipase C, TGF- β 1; transforming growth factor beta-1.

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VI. VITA

Enrique E. Rosado was born on [REDACTED] to [REDACTED] and [REDACTED] [REDACTED] in Ponce, Puerto Rico. Following graduation with honors from Ponce High School, Ponce, Puerto Rico, he attended University of Puerto Rico in Cayey, Puerto Rico where he earned a Bachelor of Science degree in 1988. That same year he joined the US Coast Guard and served as Health Services Technician at various locations in the United States. In 1993 he transferred his service to the US Coast Guard Reserve, and entered to the University of Puerto Rico School of Dentistry, San Juan, Puerto Rico. In 1996 he was awarded with the US Air Force Health Professionals Scholarship, and was commissioned as a second lieutenant in the Air Force. Dr Rosado received his Doctor of Dental Medicine degree from the University of Puerto Rico in 1997. After dental school he completed a residency in Advanced Education in General Dentistry at Eglin Regional Hospital, Eglin Air Force Base, Florida from 1997-1998. Upon completion of his duties at Goodfellow Air Force Base, San Angelo, Texas from August 1998 to May 2000, he began a three-year Air Force sponsored periodontics residency at the University of Texas Health Science Center, San Antonio, Texas and Wilford Hall Medical Center, Lackland Air Force Base, Texas. Dr Rosado is married to the former [REDACTED] from Caguas, Puerto Rico, and they have two children: [REDACTED] twelve years old, and [REDACTED] four years old.